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# THE CELLULAR ORIGIN OF BIOLUMINESCENCE IN THE COLONIAL HYDROID *OBELIA*

JAMES G. MORIN AND GEORGE T. REYNOLDS

*Department of Biology, University of California, Los Angeles, California 90024; Joseph Henry Laboratories, Department of Physics, Princeton University Princeton, New Jersey 08540; and The Marine Biological Laboratory, Woods Hole, Massachusetts 02543*

The ability to produce light is a phenomenon present in many marine organisms. Within the hydrozoan coelenterates the mechanisms that control the production of light are gradually coming to be known and understood. Using the colonial hydroid *Obelia*, Morin and Hastings (1971a, 1971b) presented biochemical information on light emission from a calcium activated photoprotein and a secondary emission from an associated green fluorescent protein via an energy transfer. Physiological mechanisms controlling the multiple flash response in *Obelia* have been examined by Morin and Cooke (1971b, 1971c). The responses were monitored by recording the electrical potentials of an excitation system and by measuring the coupled luminescence of small spots within the animal.

While these previous papers have examined the mechanisms of bioluminescence, little is known of the structure of the photogenic tissues in hydroids. In the present paper we describe some of the structural aspects of the luminescent effector cells in *Obelia*. For the work described in this paper, image intensification and fluoresence techniques were used to show that luminescent and unique green fluorescent sites are identical; and to determine the size, shape, distribution and localization of these luminescent sites. Preliminary reports of this work have been published (Morin, Reynolds and Hastings, 1968; Morin and Reynolds, 1969, 1970).

Panceri's papers (1876, 1877) are the most recent accounts of the sources of bioluminescence in the hydroid form of the hydrozoa. He concluded that luminescence was located as discrete spots in the ectodermal tissues everywhere within *Campanularia flexuosa*. Davenport and Nicol (1955) investigated the sources of luminescence in several hydrozoan medusae and showed that the photogenic material was intracellular, with masses of several thousand cells lying just under the gastrodermis of the marginal canal.

Image intensification has been used to locate the exact sources of luminescence in the firefly (Hanson, Miller and Reynolds, 1969), *Renilla* (Buck, Hanson and Reynolds, 1967), *Noctiluca* (Eckert and Reynolds, 1967), *Pyrocystis* (Swift and Reynolds, 1968), *Gonyaulax* (Reynolds, Hastings, Sato and Sweeney, 1966), and *Obelia* (Morin, Reynolds and Hastings, 1968). Fluoresence techniques have been used for the inspection of luminescent regions in *Mnemiopsis* (Harvey, 1925; Harvey and Marfey, 1958), certain annelids (Nicol, 1953, 1954), *Noctiluca* (Eckert and Reynolds, 1967), *Aequorea* and other hydromedusae (Davenport and Nicol, 1955), and in the pennatulids: *Pennatula, Pteroides, Veretillum* (Titschack, 1964, 1966), *Ptilosarcus, Renilla, Stylatula, Acanthoptilum* and *Virgularia* (Morin and Reynolds, 1970; Morin *et al.*, in preparation).

## Materials and Methods

*Obelia geniculata* (L.) was obtained and cultured according to the method of Morin and Cooke (1971a). The luminescent sites were examined using three methods: (1) autophotography by means of image intensification, (2) fluorescence microscopy and (3) histology which consisted of fixation by means of freeze-drying with subsequent embedding and sectioning for examination with the fluorescence microscope.

### *Autophotography*

Flashes of light for autophotography were evoked by applying 0.5 to 5 msec duration square pulses from a stimulator through a pair of fine, closely spaced silver wires placed across an upright of *Obelia* (Morin and Cooke, 1971b).

The physical arrangement of the image intensifier consisted of a microscope (American Optical) supplemented with a beam director (Zeiss) such that the field could be directed to the oculars for a direct view, to a camera for direct photography, or to the photocathode of the image intensifier tube for intensification and (a) photography at a second camera or (b) recorded on magnetic tape with simultaneous television monitoring. Technical details are given elsewhere (Reynolds, 1972). Magnifications of 3.5 ×, 10 × and 55 × which gave fields of 4 mm, 1.5 mm and 0.25 mm diameter respectively, were used.

Rear illumination photographs were taken by light transmitted from below the microscope stage which was directed to the image tube cathode and recorded.

Image intensification provided a direct means of photographing very low light levels. The luminescence was too weak to be recorded on film without such image intensification. The image intensifier tube gain was varied from approximately $10^4$ to $10^6$, depending on the magnification and light output of the specimen. Even with highest numerical aperture objectives used, only the order of a few per cent of the light was collected and transmitted through the microscope, so that in general, high image tube gains were required.

Sources of image noise which produced small spots on the developed film were (1) thermal electrons from the cathode, (2) background grains in the film (fog) and (3) scatter and reflection within the specimens, chamber and optical system. The large difference in size between the luminescent spots and the small noise spots reduced this problem to a negligible level (Fig. 1).

### *Fluorescence microscopy*

Fluoresence was photographed through the image tube by means of rear illumination combined with the proper filters. A blue (460 nm) interference excitation filter was placed between the light source and the specimen, and a green (507 nm) interference pass filter was placed between the specimen (after the optics) and the image tube. The resulting image with its fluorescent spots was photographed on the camera behind the image intensifier for comparison with the pictures of the luminescence taken of the same field.

Still photographs of the image tube anode were taken using an f/1.9 lens and Polaroid film (ASA 10,000). With the room totally darkened, the shutter was opened, the specimen was stimulated and then the shutter was mechanically closed

by the observer of the image tube anode, after the flash had been seen (usually 1–2 seconds).

A Leitz ultraviolet microscope with 2.5 ×, 10 ×, 54 × (oil immersion) and 94 × (oil immersion) objectives was used for fluorescence microscopy without the image intensifier. Ultraviolet illumination from a 200 watt, high pressure mercury lamp was used with a dark field condenser in order to maximize the observed fluoresence. Exciting light passed through a heat absorbing filter (Leitz BG 38) and an ultraviolet pass filter (Leitz BG 12) with a peak transmittance at approximately 400 nm. A barrier filter (Leitz K510) removed wavelengths below 500 nm. Kodachrome Tri-X black and white film was used for photography.

*Freeze-drying methods*

The freeze-drying method was similar to that of Rude (1966). The specimens were pinned to a planchet in sea water, dipped into distilled water for about one second in order to remove external salts, drained briefly on filter paper, and then plunged into isopentane cooled by liquid nitrogen (−160° C). The frozen colonies were transferred to a freeze-dry appartus and dried for about three days at a pressure of 2 $\mu$ Hg and an outside temperature of −40° C. The specimens were then slowly brought to room temperature. The specimens were placed in Maraglas embedding medium in a vacuum desiccator for several hours; they were then transferred to a 40° C oven for about seven hours until the Maraglas hardened. Seven $\mu$ serial cross sections were made on a Spencer A.O. microtome with metal knives. The serial sections were mounted in Entellan, a non-fluorescing mounting medium, on microscope slides and examined with the fluorescence microscope.

## Results

*Visual appearance of the luminescence*

Stimulation of *Obelia geniculata* evoked light which emanated from small points within the colonies. Within a given microscope field the light showed distinct multiple flashes in response to individual stimuli. It was difficult to determine visually the precise source of the luminescence because of the flickering and relatively weak light. It was not possible by visual means to be certain if individual spots were flickering or if the flicker was a consequence of sequential luminescence of different spots, possibly along the length of the colony.

Evidence, presented from photometric responses of single luminescent sites (shown by fluorescence), indicated that single spots did flash repetitively (Morin and Cooke, 1971b, 1971c). Autophotography and fluorescence microscopy provided further evidence for repetitive flashing of single spots and information about the general location of the luminescent sites.

*Autophotography and fluorescence of the luminescent sites*

*General characteristics of the luminescent sites.* The usual pattern of luminescence in *Obelia geniculata,* demonstrated by single frame autophotography, is shown in Figure 1. Figure 1A-C shows a 3.5 × field of an upright with (A) rear

illumination, (B) luminescence and (C) both almost superimposed. An enlarged field (10 ×) of the central portion of figure 1A-C is shown in Figure 1D-F with the same format. The photographs show that the luminescent sites are discrete spots of variable size. The spots are located in the uprights and pedicels and not in the hydranths; the spots remain fixed in their spatial patterns, at least between successive photographs spaced several minutes apart. In addition to this constant

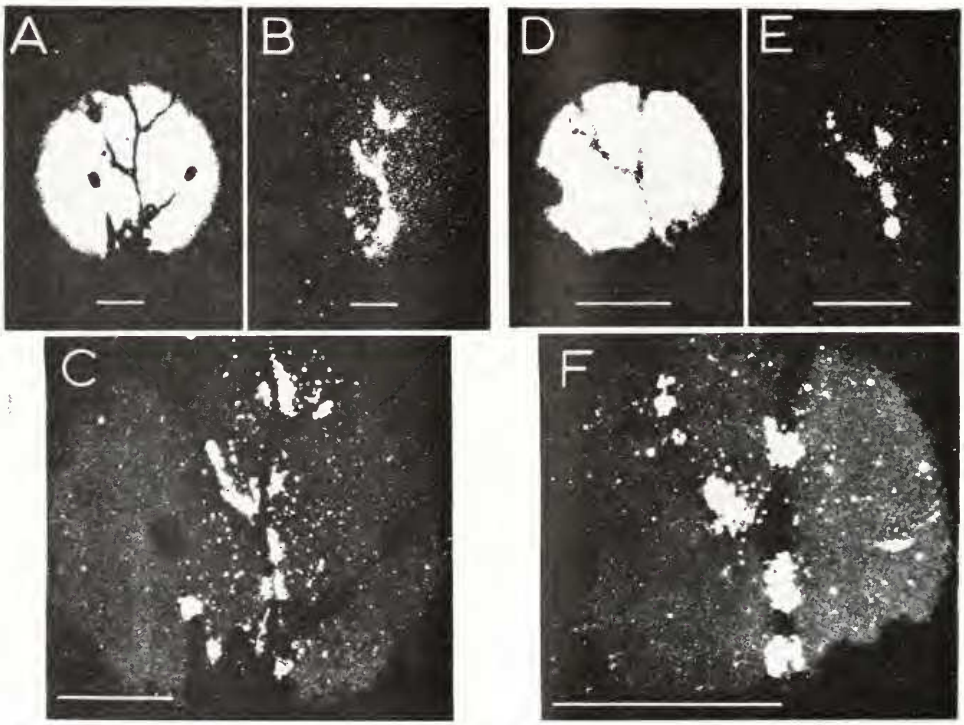


FIGURE 1. Image intensifier photographs of *Obelia geniculata* bioluminescence: (A–C). Low power (3.5 ×) photographs showing (A) rear illumination of upright, (B) autophotograph of upright luminescence and (C) superposition of rear illumination (A) and autophotograph (B). (D–F), same colony but at a higher magnification (10 ×) taken a few minutes after (A–C); same format. Bars indicate 1 mm. Scattered small spots in (B) and (E) are image noise (see text for details).

pattern of organization, other photographs showed that the stolons contain luminescent spots and that medusae within the gonangia show no luminescence upon stimulation of the colony, although mature medusae are capable of emitting light.

*Correspondence of fluorescence with luminescence.* Biochemical evidence indicated that the luminescent sites could be located and observed using a method which exploits the fluorescence charcteristics of the emission system when excited with blue light (Hasting and Morin, 1969a, 1969b; Morin and Hastings, 1971b). Such a method involved excitation of the luminescent sites with 460 nm light and examination of the emitted light using a barrier filter to exclude wavelengths shorter

than 500 nm. The sites revealed by this method fluoresced a bright green color ($\lambda_{max} = 508$ nm). Yellow-green fluorescence from small particles (a few microns) was also observed within the colonies, especially in well nourished ones. There was no correspondence between these latter fluorescent sites and the luminescent sites. Epiphytic diatoms attached to the perisarc of the colonies displayed the characteristic red color of chlorophyll fluorescence.

The direct correspondence between luminescence and fluorescence was shown by successive photographs of the same field through the image tube. First, an autophotograph was taken of the luminescence (Fig. 2A) and then a photograph was taken of fluorescence excited by filtered rear illumination (Fig. 2B). In all cases there was an exact correspondence for each site. In the following sections, therefore, it is considered that observations of the fluorescent sites provide a description of the luminescent sites.

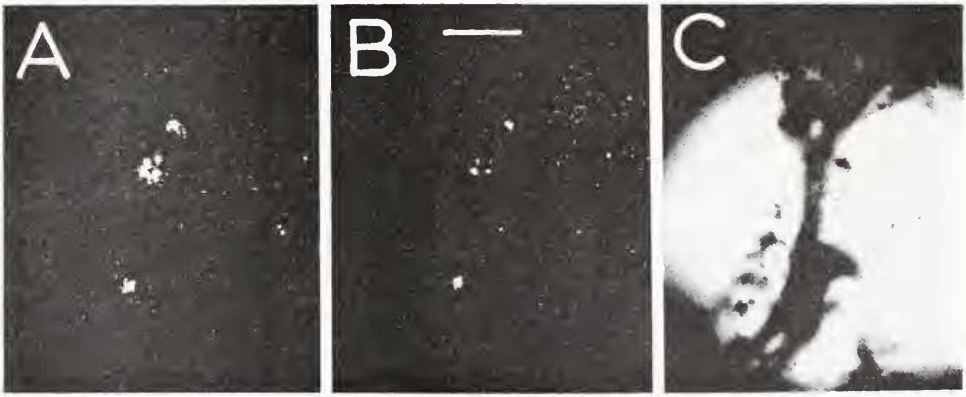


FIGURE 2. Autophotographic (A), fluorescence (B) and rear illumination (C) pictures of an *Obelia* upright. Note the direct correspondence between the luminescent and fluorescent spots (six in each). They directly superimpose. The scale bar indicates 200 $\mu$.

The fluorescence method is extremely useful for characterizing the luminescent sites because the fluorescent emission is not intermittent, as is the luminescence, and the living material can be examined without any manipulation of the colonies (*i.e.*, stimulation, surgical or histological procedures).

*Dimensions and shape of the luminescent sites.* The size of the luminescent sites as shown by fluorescence varies within the range of 5 to 30 $\mu$ with a usual size of about $10 \times 20$ $\mu$. The sites shown by autophotography are slightly larger than fluorescence sites possibly because of overexposure. The nonfluorescent cells in the same regions as the fluorescent sites have dimensions similar to those of the fluorescent sites. This observation strongly suggests that the luminescent sites are single cells, and we will therefore refer to them as *photocytes*. Histological evidence given below supports this conclusion.

The photocytes possess a wide range of shapes (Fig. 3). They are best observed in very young, distal tissues or in the distal part of the pedicels where the perisarc is relatively transparent and not overgrown by epiphytes (Fig. 3B).

A characteristic feature of these cells is the frequent occurrence of one or

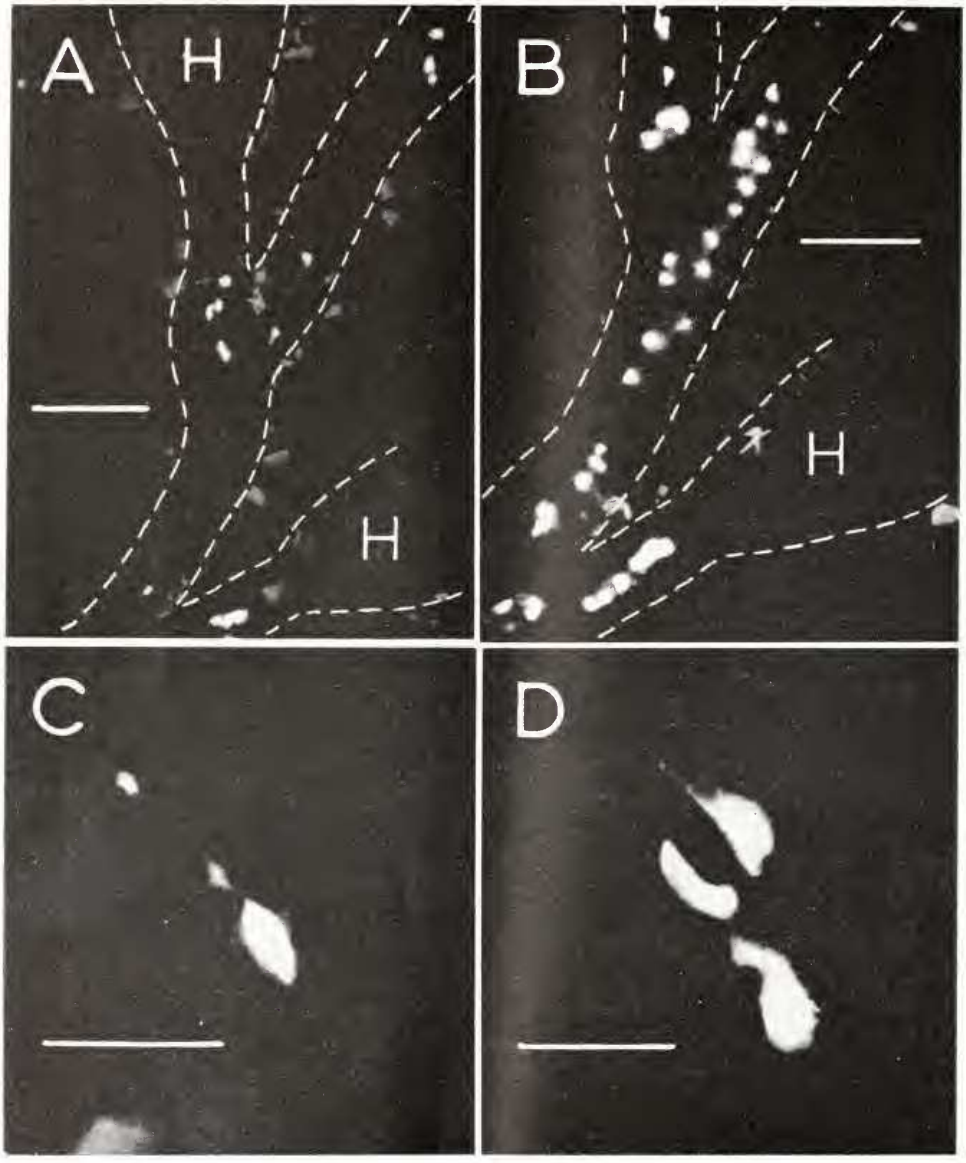


FIGURE 3. Fluorescence microscope photographs of living *Obelia geniculata*. (A). Basal part of an upright showing three nodes; each node and pedicel shows a cluster of photocytes. The skeletal outline of the hydroid has been dotted in white. Hydranths are indicated by an H. The dim fluorescent material around the photocytes are red fluorescing diatoms attached to the hydroid. (B). Distal part of an upright showing two nodes; note the more numerous photocytes than in (A) and the limited diatom growth. Again the outline has been dotted in. (C). Single photocyte in a pedicel; note the long projection. (D). Three photocytes with projections; note the dark presumed nucleus of the upper cell. Bar indicates 40 $\mu$ in (A) and (B); 25 $\mu$ in (C) and (D).

more cytoplasmic projections that may be up to 20 $\mu$ in length (Fig. 3C, D). These projections have no apparent orientation to neighboring photocytes. The distance between photocytes varies from several mm, usually in the stolons or proximal uprights (Fig. 3A), to near contact, especially in the pedicels and distal parts of the uprights (Fig. 3B).

The distribution of fluorescence within a photocyte including the projections usually appears homogeneous in a living whole mount except for a nonfluorescent

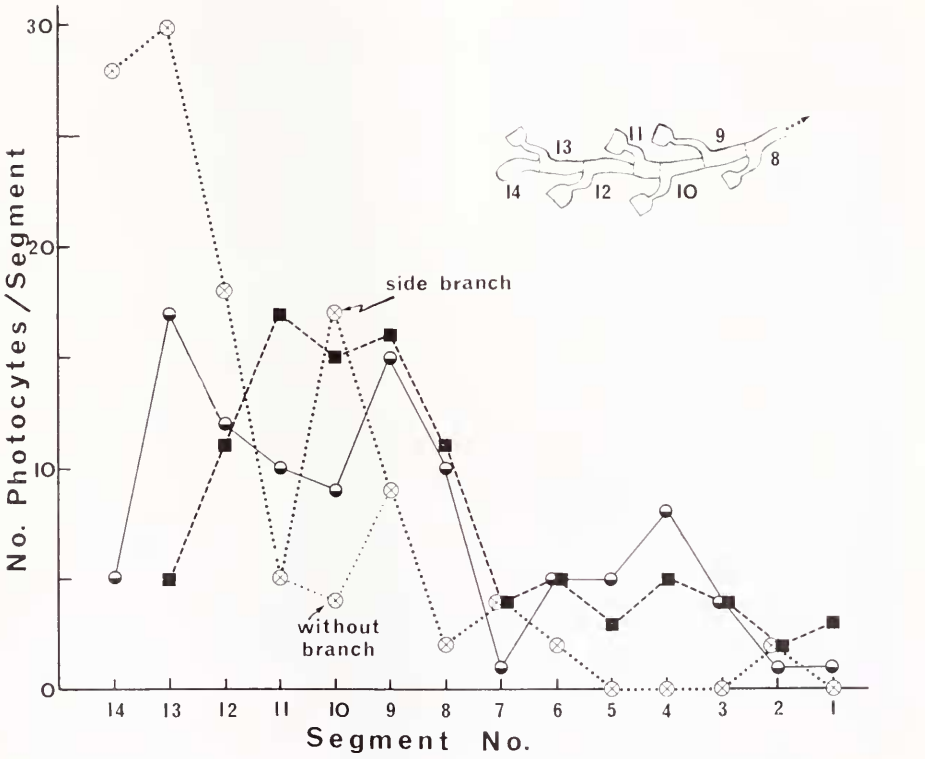


FIGURE 4. The number of photocytes per "segment" (one "segment" as defined here includes the pedicel, node and proximal internode—see diagram, upper right) is plotted against the "segment" number (the oldest, most proximal "segment" is referred to here as the first "segment") for three different, randomly selected uprights. One (circle with a cross) shows the number when a small side branch was and was not considered in the count.

inclusion (sometimes two) within each cell. The size and location of this inclusion is suggestive of a nucleus (Fig. 3D). Occasionally a compartmentalization of the fluorescence within the photocytes is indicated.

*Distribution and density of the photocytes.* The general pattern of photocyte distribution and density is shown in the photographs of fluorescence in Figure 3A, B. These photographs show that (1) there are more photocytes in the pedicels and nodes than in the internodes of the upright, (2) the photocytes are all of approximately the same size and (3) there is a greater concentration of photocytes

in distal parts of the upright. Also a short side branch is usually marked by a concentration of photocytes, while long side branches show a distribution similar to the primary upright.

The number of photocytes per "segment" (one "segment" as defined here includes a pedicel, node and proximal internode) in uprights from three different colonies is plotted in Figure 4. The variation is considerable, but the generalization that photocytes are more concentrated distally is supported.

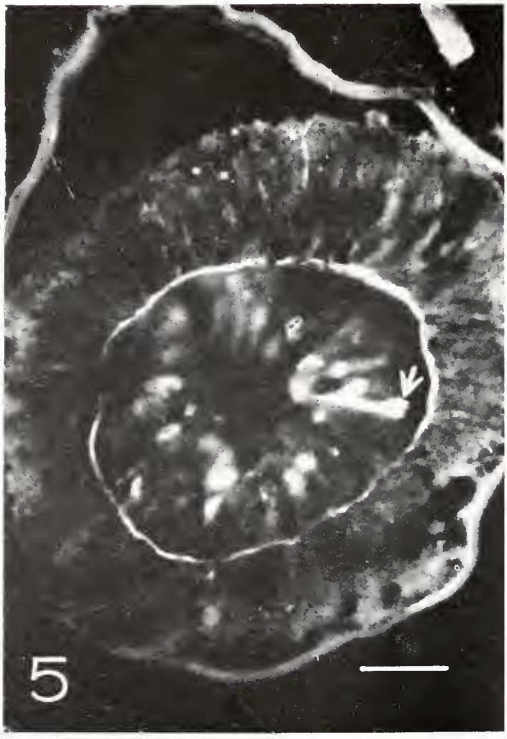



FIGURE 5. Fluorescence microscope photograph of a freeze-dried cross-section of a portion of an upright containing a photocyte (arrow) in the endoderm. Note the long projection of the photocyte toward the coelenteron (central hollow). The endoderm, ectoderm and perisarc are easily distinguished. Scale bar indicates 20 $\mu$.

FIGURE 6. Fluorescence microscope photograph of a living *Obelia bicuspidata* showing the concentration of photocytes at the tip of the pedicel. Bar indicates 100 $\mu$.

Intermittent examination of an upright over a period of several hours showed that the photocytes did not move a visible distance in that time.

*Location of photocytes within the endoderm.* The observations of living whole mounts indicated that the cells do not lie in the relatively transparent ectoderm (Fig. 3A, B). Fluorescence microscopic examination of freeze-dried, serial sectioned material showed that the photocytes are located in the endoderm (Fig. 5). A reconstruction from serial sections showed a correspondence of the green fluorescent cells seen histologically to the photocyte positions shown in a fluorescence

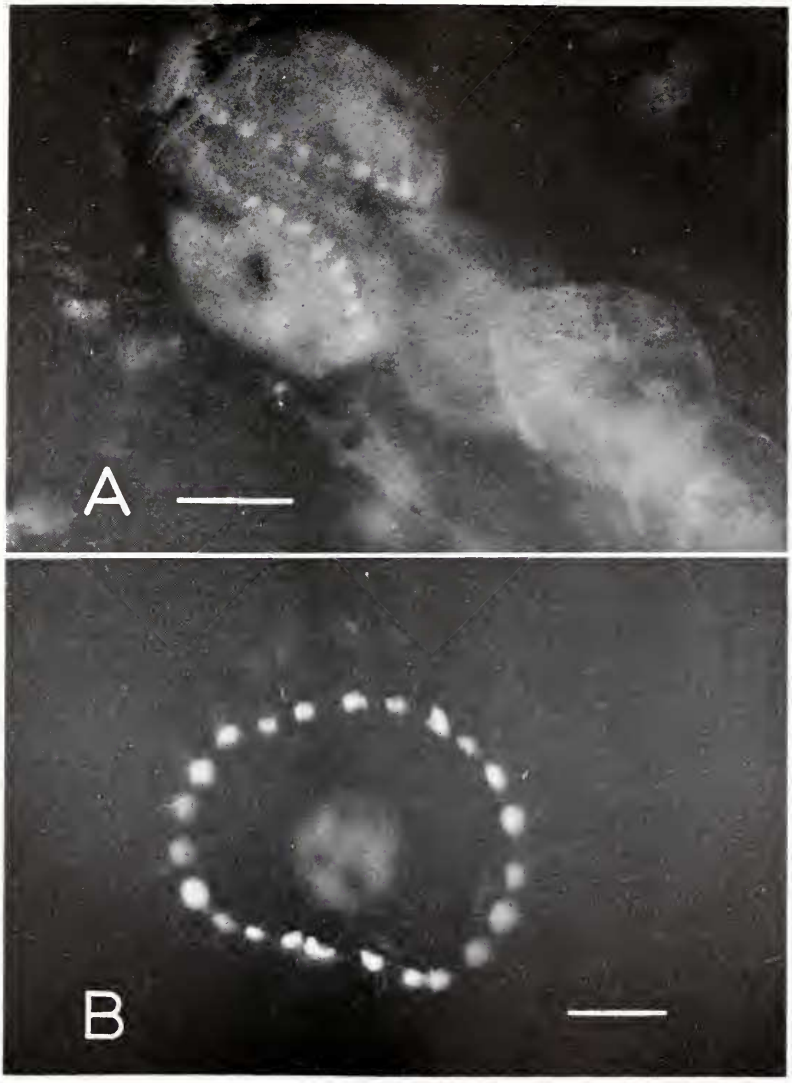


FIGURE 7. (A). Fluorescence microscope photograph of a gonangium containing developing medusae. The most mature medusae (containing the most fluorescence) are toward the left (near the opening of the gonangium). (B). Fluorescence microscope photograph of a newly freed medusa showing the distribution of green fluorescence in the tentacular bulbs; the manubrium shows a yellowish fluorescence and is not luminescent. Scale bars indicate 100 $\mu$. (Living whole mounts).

microscope photograph taken earlier of the same specimen as a living whole mount. Freeze-dried materials showed more fluorescence of the other tissues than did the living material. This greater fluorescence tended to obscure the green fluorescence of the photocytes to a slight degree (Fig. 5).

The bulk of the photocyte, presumably the soma, is usually adjacent to the mesoglea (Fig. 5). Cytoplasmic projections often extend in several directions but not toward the ectoderm. One projection almost always extends to the coelenteron (Fig. 5). Photocytes were never seen in ectodermal tissues. No cases were observed where direct contact occurred between two photocytes. In the freeze-dried sections of photocytes the fluorescent material often appears to be particulate or granular. Since living photocytes appear homogeneous, this granularity may be a fixation artifact.

*Fluorescence of the gonangium and the medusa*

Although no luminescence following colony stimulation was observed in gonangia in the few cases examined with image intensification (performed prior to the discovery of the fluorescence method), fluorescence microscopy revealed that occasionally photocytes occur within the blastostyle of the gonangium. Visual observations indicated that these photocytes do luminesce upon colony stimulation.

The youngest, developing (proximal) medusae show no fluorescence. More distal medusae show a weak ring of disjunct green fluorescent spots near the ring canal (Fig. 7A). The intensity of the ring of spots, which number sixteen, increases with the age of medusae. Visual observations indicated that these attached medusae do not luminesce upon stimulation of the hydroid colony. Newly released medusae show fluorescence and luminescence at the site of the tentacular bulb at the base of each tentacle (Fig. 7B). As judged from microscopic observations, medusae produce luminescence from these sites upon stimulation with isotonic KCl or direct electric shocks.

The distribution of fluorescent sites is similar to the pattern of luminescence in the hydromedusae *Halistaura* (Davenport and Nicol, 1955).

*Distribution of luminescence and green fluorescence among the hydrozoa*

It has been previously reported that *Obelia bicuspidata, O. commisuralis, O. longissima, Clytia edwardsi* and *Campanularia calceolifera* showed photometrically recorded flashing responses similar to *O. geniculata* (Morin and Cooke, 1971b). *O. commisuralis* and *C. calceolifera* were examined by means of the image intensifier and proved to have a distribution of luminescence similar to but less dense than that of *O. geniculata.* Fluorescence observations also demonstrated a similar pattern but with fewer photocytes than in *O. geniculata. Clytia edwardsi* shows a greater density of photocytes than in *O. geniculata* by both methods. On the other hand both methods show that the photocytes in *Obelia longissima* and *O. bicuspidata* are concentrated at the tip of the pedicel immediately below the diaphragm of the hydranth (Fig. 6). Using fluorescence methods only, a similar concentrated photocyte distribution was observed in *Lovenella gracilis.*

Several other hydroids were examined photometrically for luminescence using isotonic KCl as the stimulus, and most were also examined for green fluorescence using fluorescence microscopy. In all the cases examined the luminous forms showed green fluorescence while the nonluminous forms showed no such fluorescence (Morin, in preparation). Green fluorescent cells were not found in the hydranths of any species examined. However, a photocyte was observed in the

base of the hydranth body in *O. geniculata* in a few cases (less than 0.1% of the hydranths examined).

To date only the families Campanulariidae and Campanuliniidae have been found to possess luminescent hydroid members and not all species within the families are luminescent.

## Discussion

The discovery of the fluorescent properties of the photocytes in *Obelia geniculata* has proven to be extremely useful in characterizing the photocyte both structurally and physiologically.

The colonies need not be physically manipulated in any way other than by illumination with a deep blue (460 nm) light in order to see the photocytes. This method, therefore, should be of use to the developmental biologist who is interested in coelenterates, because it allows the study of an effector, dependent on a through-conducting excitation system, which can be monitored at any time without disturbing the colony.

That the fluorescence is maintained within the photocyte even following freeze-drying and embedding is a promising indication that at least mild staining and fixing techniques may be used without destroying the fluorescence and, therefore, that this procedure will ultimately lead to a detailed study of the ultrastructural characteristics of these photocytes.

Titschack (1964, 1966) has also observed this particular luminescent associated green fluorescence in the alcyonarian coelenterates, *Pennatula, Peteroides* and *Veretillum*. We have also reported a similar fluorescence corresponding to luminescence in several hydroids (*Campanularia, Clytia, Obelia, Lovenella, Aequorea* and *Phialidium*) and in the alcyonarians *Renilla, Ptilosarcus* and *Stylatula* (Morin and Reynolds, 1970). The biochemical mechanism responsible for this coupling between the bioluminescence and the green fluorescence have been partially established for a number of hydrozoans and anthozoans (Morin and Hastings, 1971b; Cormier, Hori, Karakanis, Anderson, Wampler, Morin and Hastings, 1973; Wampler, Karkhanis, Morin and Cormier, 1973).

The location of the luminescence in *Obelia geniculata* and all other luminescent hydroids that we have examined is at variance with the conclusions made by Panceri (1876, 1877) on the basis of his study of *Campanularia flexuosa*. He believed that the luminescence resided in the epidermis of all parts of the hydroid including the hydranths. Our results, however, show conclusively that the luminescence is endodermal within only the pedicels, stems and stolons and not in the hydranths. We have performed an approximate duplication of Panceri's experimental procedures of observing a colonly of *Obelia geniculata* after addition of fresh water and find the luminescence too weak for definite visual identification of the cell layer, even with rapid changing from a non-illuminated to an illuminated condition. Furthermore, the tissues began to disintegrate within a few seconds after the fresh water was added. The whole colony became progressively more fluid and disorganized so that anatomical orientation became impossible especially in the hydranth region where the exposure to water first occurs. These observations make Panceri's conclusions open to doubt.

Endodermal localization of bioluminescent cells has also been shown for other coelenterates including the hydromedusan *Aequorea* (Davenport and Nicol, 1955), some pennatulids (Titschack, 1966) and the ctenophore *Mnemiopsis* (Freeman and Reynolds, 1973).

The locations of the bioluminescence in *Obelia* as revealed by autophotography and fluorescence microscopy are curious. Luminescence is located in the least exposed region of the colonies: in the innermost endodermal tissue layer beneath the ectoderm and the skeletal perisarc. The adaptive significance of this peculiar anatomical organization is obscure.

It is interesting to consider the distribution of the photocytes in light of the relative longevity of the tissues. Colonies undergo a regression-replacement cycle in which the hydranth is resorbed into the colony and replaced by a new hydranth. This cycle occurs about once every six or seven days (Crowell, 1961). However, the coenosarc within the pedicels, uprights and stolons does not show such a cycle. During adverse conditions all the hydranths in a colony may regress, and the colony can live for several weeks in this condition. The photocytes are found only in the relatively more stable tissues of the coenosarc, and they are not found in the short lived hydranth tissues. In addition, the density of the photocytes is greatest near the actively growing areas such as the tip of an upright or the site of a side branch.

The photocytes have been shown to be coupled to an excitation system, the luminescent potential (LP) system (Morin and Cooke, 1971b, 1971c). The endodermal position of the photocytes indicates that the LP system is probably also endodermal. It has been shown that another conducting system, the rhythmic potential (RP) system, in *Hydra* is situated within the endoderm (Shibley, 1969).

At present there is no evidence available that shows morphological connections between the photocytes and other cells. The photocytes, at least at the level at which they have been examined, appear to be specialized for a single function: luminescence. It will be of interest to discover whether these cells show multiple functions such as is found in the epitheliomuscle cells of the ectoderm and digestive cells of the endedorm both of which contain myofibrils.

A few generalizations on the systematic distribution of luminescence within the hydroids can be made from our studies that refine those presented by Harvey (1952): (1) There are no known luminescent gymnoblastic hydroids. (2) Only the families Campanulariidae and Campanuliniidae of the Calyptoblastea definitely have luminescent polypoid members. (3) Both these families have luminescent and non-luminescent species. (4) In at least some of the species both the polypoid (hydroid) and medusoid forms are luminescent.

## Summary

1. *Obelia geniculata* luminescent sites were examined using image intensifier autophotography and fluorescence microscopy techniques.

2. Autophotography and superimposed fluorescence showed that luminescence emanates from discrete spots located within the pedicels, uprights and stolons of the colonies. The green fluorescent sites correspond to luminescent sites.

3. The size of the spots averages about 10 to 20 $\mu$. They are considered to be cells and are termed *photocytes*.

4. The photocytes are concentrated distally in an upright, are more abundant in pedicels and nodes than in the internodes, and are most abundant in actively growing regions.

5. The photocytes are located in the endoderm against the mesoglea, and they have cytoplasmic projections up to 20 $\mu$ long; one of these projections usually extends to the coelenteron.

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